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1 **Antifungal activity of fermented dairy ingredients: identification of antifungal**
2 **compounds.**

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25 **Keywords: antifungal; fermentate; organic acids; fatty acids; volatile compounds**

26 **Abstract**

27 Fungi are commonly identified as the cause for dairy food spoilage. This can lead to
28 substantial economic losses for the dairy industry as well as consumer dissatisfaction. In this
29 context, biopreservation of fermented dairy products using lactic acid bacteria,
30 propionibacteria and fungi capable of producing a large range of antifungal metabolites is of
31 major interest. In a previous study, extensive screening was performed *in vitro* and *in situ* to
32 select 3 dairy fermentates (derived from *Propionibacterium jensenii* CIRM-BIA1774,
33 *Lactobacillus rhamnosus* CIRM-BIA1952 and *Mucor lanceolatus* UBOCC-A-109193,
34 respectively) with antifungal activity. The aim of the present study was to determine the main
35 compounds responsible for this antifungal activity. Fifty-six known antifungal compounds as
36 well as volatiles were targeted using different analytical methods (conventional LC and GC,
37 GC-MS, LC-QToF). The most abundant antifungal compounds in *P. jensenii*-, *L. rhamnosus*-
38 and *M. lanceolatus*-derived fermentates corresponded to propionic and acetic acids, lactic and
39 acetic acids, and butyric acid, respectively. Many other antifungal compounds (organic acids,
40 free fatty acids, volatile compounds) were identified but at lower levels. In addition, an
41 untargeted approach using nano LC-MS/MS identified a 9-amino acid peptide derived from
42 α_{s2} -casein in the *L. rhamnosus*-derived fermentate. This peptide inhibited *M. racemosus* and
43 *R. mucilaginosa in vitro*. This study provides new insights on the molecules involved in
44 antifungal activities of food-grade microorganism fermentates which could be used as
45 antifungal ingredients in the dairy industry.

46

47 **1. Introduction**

48 Fungi can cause spoilage of dairy products. Indeed, due to their intrinsic characteristics (e.g.
49 acidic pH and intermediate water activity, nutrient composition), dairy products constitute a
50 favorable environment for yeast and mold growth (Rohm et al., 1992). Fungal spoilage leads
51 to food waste and substantial economic losses, and can also impact brand image in the eye of
52 the consumer (Pitt and Hocking, 2009). The most common fungi involved in dairy product
53 spoilage belong to *Penicillium*, *Mucor* and *Cladosporium* genera for moulds and *Candida*,
54 *Kluyveromyces* and *Yarrowia* for yeasts (Garnier et al., 2017; Pitt and Hocking, 2009).

55 Control of fungal spoilers in the dairy industry is therefore of utmost importance. Today,
56 different preventive and control methods (also known as “hurdle technologies”) are
57 implemented and combined to prevent contamination during product manufacturing but also
58 to inhibit or slow down growth of fungal spoilers. These methods include, for example, use of
59 good manufacturing and hygiene practices, implementation of the hazard analysis and critical
60 control points (HACCP) system, air filtration and packaging in aseptic conditions, heat
61 treatment, refrigeration and salting/brining. In addition, chemical preservatives can be used
62 and extend dairy product shelf-life with respect to relevant legislation (Garnier et al., 2017).
63 However, regarding chemical preservatives, consumers are more frequently requesting
64 preservative-free products. “Natural” preservation alternatives, such as biopreservation, are
65 therefore increasingly used (Leyva Salas et al., 2017).

66 Lactic acid bacteria (LAB), propionibacteria and fungi are good candidates for
67 biopreservation as they have a long history of safe use in human consumption. LAB are used
68 in various dairy products such as yogurt, cheese or kefir (Corrieu and Luquet, 2008). As a
69 consequence, some LAB, but also propionibacteria species, are used as bioprotective agents
70 (as cultures or antifungal ingredients) in dairy products. Commercial antifungal solutions such
71 as Holdbac (DuPont Danisco, Dangé, Saint Romain, France), FRESHQ (CHR Hansen,

72 Hovedstaden, Denmark) and Natamax (DuPont Danisco, Dangé, Saint Romain, France)
73 protective cultures containing LAB species and/or propionibacteria, as well as antifungal
74 ingredients such as MicroGARD, derived from Propionibacteria species, are currently
75 available on the market (Leyva Salas et al. 2017). These microorganisms are able to produce a
76 large spectrum of compounds such as organic acids, fatty acids (Sjögren et al., 2003), cyclic
77 dipeptides (Ström et al., 2002) and proteinaceous compounds (Rizzello et al., 2011) that are
78 potentially involved in antifungal activities, acting in synergy (Crowley et al., 2013; Leyva
79 Salas et al., 2017). Concerning fungal biocontrol agents, they are mostly used in fruit
80 preparations or fermented meat (Leyva Salas et al., 2017). Studies have shown their **ability** to
81 produce antifungal compounds such as killer-toxins, organic acids or peptides (Acosta et al.,
82 2009 ; Coda et al., 2013 ; Ignatova et al., 2015 ; Leyva Salas et al., 2017; Nally et al., 2015).
83 When it comes to dairy product biopreservation, only a few studies have dealt with the action
84 mechanisms of bioprotective cultures (Crowley et al., 2013). In a recent study, Mieszkin et al.
85 (2017) explored the action mechanisms of *Lactobacillus harbinensis* K.V9.3.1.Np against
86 *Yarrowia lipolytica* in fermented milk. The observed fungistatic effect of the tested
87 bioprotective culture involved different organic acids and led a decrease in intracellular pH
88 and membrane depolarization. More recently, Leyva Salas et al. (2019), using different
89 analytical tools targeting 56 known antifungal compounds and volatile compounds,
90 highlighted the diversity of antifungal molecules produced by different bioprotective cultures
91 in various dairy products.

92 In another recent study (Garnier et al., 2019), 3 ingredients corresponding to dairy microbial
93 fermentates with antifungal activity were selected after *in vitro* screening. Their efficiency
94 was further validated in real dairy products (*i.e.* after incorporation in sour cream and surface-
95 spraying on semi-hard cheese). The aim of the present study was to investigate which
96 compounds were responsible for the antifungal activity in the previously selected fermentates.

97 To do so, different analytical methods, namely conventional LC and GC, GC-MS, LC-QToF
98 and nano-LC-MS/MS, were applied, to detect and quantify antifungal metabolites, including
99 56 organic and fatty acids (targeted approach), and volatile compounds and peptides (non-
100 targeted approach).

101

102 2. **Material and methods**

103 2.1. Fermentate preparation

104 Fermentates with antifungal activity were selected from a previous study (Garnier et al.,
105 2019). They were obtained from 2 fermented dairy media: a reconstituted 10% low heat milk
106 supplemented with 45% anhydrous milk fat (for the *Lactobacillus rhamnosus* CIRM-
107 BIA1952 and *Mucor lanceolatus* UBOCC-A-109119 strains) and an ultrafiltration permeate
108 supplemented with 1% yeast extract (for the *Propionibacterium jensenii* CIRM-BIA1774
109 strain). They were kept as a lyophilisate for *P. jensenii* CIRM-BIA1774 and *L. rhamnosus*
110 CIRM-BIA1952 and as a 0.45 µm filtered culture supernatant for *Mucor lanceolatus*
111 UBOCC-A-109119, as previously described (Garnier et al., 2019). For each experiment, three
112 biological replicates of each fermentate were analyzed.

113

114 2.2. Identification and quantification of potential antifungal compounds

115 2.2.1. Antifungal compounds identification by LC-QToF

116 2.2.1.1. Standard preparation

117 Thirty-one compounds with known antifungal activity were searched for as described
118 previously (Brosnan et al. 2014; Le Lay et al., 2016) except that 5 additional molecules were
119 added, namely, 4-di-tert-butylphenol, mevalonolactone, N-acetyl-D-glucosamine, phenyl
120 acetate and ricinoleic acid (Leyva Salas et al., 2019). Standards were prepared by mixing all
121 compounds at different concentrations. Individual stock solutions at 5 mg/mL were prepared

122 in water or acetonitrile, and mixed together to obtain a standard mix at 100 ppm in H₂O/ACN
123 (90/10, v/v). A matrix-matched calibration curve was built by diluting the standard mix in a
124 blank extract of acidified semi-skimmed milk (adjusted to pH 5 with lactic acid) at the
125 following concentrations: 1 ppm, 5 ppm, 10 ppm, 30 ppm and 50 ppm. Standards were kept at
126 -20 °C in amber vials.

127

128 2.2.1.2. Sample preparation

129 Compound extraction from fermentates (lyophilized or filtered supernatant) were performed
130 as described by Brosnan et al. (2014) with slight modifications. First, 10 mL of either
131 lyophilized fermentate resuspended in sterile water (1:10, w/v) or of supernatant were mixed
132 with 4 g of MgSO₄, 1 g of NaCl and 10 mL ethyl acetate supplemented with 1 % of formic
133 acid. The mixture was then vigorously shaken before centrifuging for 10 min at 8500 g at 4
134 °C. The organic phase was transferred into a dSPE tube (dispersive Solid Phase Extraction,
135 Agilent technologies), vortexed and centrifuged for 10 min at 2600 g. The liquid phase was
136 then recovered in a 15 mL tube, mixed with 100 µL of DMSO and evaporated under nitrogen
137 gas. The remaining 100 µL were supplemented with 900 µl of H₂O/acetonitrile (90/10, v/v),
138 filtered at 0.22 µm and stored in amber vials at -20 °C until analysis.

139

140 2.2.1.3. LC-QToF analysis and method validation

141 Detection and quantification were performed on a 1260 Infinity binary HPLC and a 6530
142 Accurate Mass LC-QToF LC/MS (Agilent Technologies) as described by Le Lay et al. (2016)
143 and Leyva Salas et al. (2019). Compounds separation was done on a Zorbax Extend-C18
144 column (201 x 150 mm, 5 µ) equipped with a pre-column (2.1 x 12.5 mm, 5 µ) (Agilent
145 technologies) and the mass spectrometer operated in negative electrospray ionization.

146 Analyses were performed under the conditions described by Le Lay et al. (2016) except that
147 injected volumes were 10 and 50 μL . Standards and samples were injected in triplicate.
148 To validate the method, extraction recovery was determined on acidified semi-skimmed milk
149 spiked with 10 ppm of each compound. Standard mix at 10 ppm in blank extract was injected
150 3 times a day for 5 consecutive days to assess the intra- and inter-day repeatability. For each
151 compound, the limit of detection (LOD) and limit of quantification (LOQ) were determined
152 based on the standard deviation of the analyte response and the standard deviation slope (ICH
153 Harmonized Tripartite Guideline, 2005)

154

155 2.2.2. Antifungal organics acid quantification by HPLC

156 Nine mL of fermentate supernatant or 3 g of lyophilized fermentates dissolved in sterile water
157 (1:2) were centrifuged for 30 min at 10 000 g. The liquid phase was half diluted in 5 mM
158 sulfuric acid, vigorously mixed and stored overnight at -20°C . After thawing, samples were
159 once again centrifuged at 10 000 g for 30 min, the upper phase recovered and filtered on 0.45
160 μm PTFE membrane. Analysis was performed as previously described (Leyva Salas et al.,
161 2019) on a HPLC Dionex system (Sunnydale, CA, USA) equipped with an Aminex HPX-87H
162 column (Biorad, Hercules, CA, USA), using 5 mM H_2SO_4 as the mobile phase, and UV (210
163 nm) and refractometer detectors. Identification and quantification were achieved using
164 standard solutions of the following organic acids at concentrations ranging from 0 to 1
165 mg/mL: acetic acid, benzoic acid, citric acid, lactic acid, propionic acid, succinic acid and 2-
166 pyrrolidone-5-carboxylic acid.

167

168 2.2.3. Free fatty acid identification by gas chromatography

169 2.2.3.1. Standard preparation

170 Identification and quantification of free fatty acids, as described by Leyva Salas et al. (2019),
171 was adapted from the method by Jong and Badings (1990). They were performed with
172 different external standards, including acetic, propionic, butyric, valeric, isovaleric, caproic,
173 enanthic, caprylic, pelargonic, capric, undecyclic, lauric, myristic, palmitic, margaric, stearic,
174 oleic, linoleic and octadecadienoic acids, with concentrations ranging from 5 to 500 µg/g. An
175 internal standard, to take into account the extraction yield, was used (0.5 mg/g of valeric acid
176 (C5:0), tridecanoic acid (C13:0) and margaric acid (C17:0) diluted in 30 mL of heptane).

177

178 2.2.3.2. Fatty acid extraction

179 Free fatty acid extraction was performed with 1 g of lyophilized fermentate or 1 mL of
180 supernatant, 3 g of anhydrous sodium sulfate (Na₂SO₄) and the mixture was ground. Then, 0.3
181 mL of H₂SO₄ at 2.5M, 1 mL of internal standards and 15 mL of ether/heptane (1:1) were
182 added before centrifugation (3 min at 100 g). The organic layer was recovered with 100 mL of
183 Na₂SO₄ to capture residual water. The pellet was resuspended in 15 mL of ether/heptane (1:1)
184 and centrifuged (3 min at 100 g). This step was repeated 3 times by pooling the organic layer.
185 Free fatty acid purification was performed with a Manifold, using a SPE 500 mg column
186 (Phenomenex, UK) conditioned with 10 mL heptane. The organic layer was transferred onto
187 the column and 10 mL heptane/2-propanol (3:2) were added for neutral lipids removal. Free
188 fatty acids were eluted with 5 mL ether diethyl with 2% formic acid and transferred in an
189 amber vial and stocked at -20°C until analysis. Samples were injected in triplicate.

190

191 2.2.3.3. Analysis of fatty acids by GC

192 Fatty acid analysis was performed by gas chromatography (Varian CP-3800) equipped with a
193 flame ionization detector (FID) and a capillary column (BP21 25m x 0.53 mm, layer 0.5 µm,

194 JW Scientific, Folsom, USA) as previously described (Leyva Salas et al., 2019). Vector gas
195 was dihydrogen at 9.7 mL/min. A temperature gradient was applied from 65°C to 240°C with
196 a ratio of 10°C/min then 10 min at 240°C using direct injection. Quantification was performed
197 using standard calibration curves with the Star Varian (version 5.3) software. A correction of
198 possible free fatty acid losses during extraction was performed with internal standards using
199 the following calculation:

200

$$201 \text{ Free fatty acid } (\mu\text{g}) = \frac{([\text{Calculated concentration of free fatty acid}] * [\text{Standard Y } (\mu\text{g})])}{[\text{Calculated concentration of standard Y}]}$$

202

203 2.2.4. Volatile compounds identification by GC-MS

204 2.2.4.1. Preparation of standards for GC-MS analysis

205 Standard compounds were used to generate standard curves and to check the response of the
206 HS-trap GC-MS system during the sample analyses. Two solutions of standard compounds
207 were prepared: one with neutral volatiles and another with short-chain fatty acids. Neutral
208 standard compounds included 4 esters (ethyl acetate, ethyl propanoate, ethyl butanoate and
209 ethyl hexanoate), 2 aldehydes (3-methylbutanal and benzaldehyde), 2 ketones (2-heptanone,
210 2,3-butanedione), 1 sulphur compound (dimethyl disulfide) at concentrations ranging from 5
211 to 1200 ng/g and 1 amyl alcohol (3-methylbutanol) at concentrations ranging from 260 to
212 50,000 ng/g. For short-chain acids, the standard mix 46975-U (Supelco, Sigma-Aldrich,
213 Saint-Louis, USA) containing acetic, propanoic, butanoic, 2-methylpropanoic, pentanoic, 3-
214 methylbutanoic, hexanoic, 4-methylpentanoic and heptanoic acids at 10mM each was used to
215 prepare standard solutions at concentrations ranging from 20 to 1000 ng/g. The pH of these
216 standard acid solutions was adjusted to 6.35±0.15 with NaOH.

217

218 2.2.4.2. Extraction of volatile compound using headspace trap

219 Extraction of volatile compounds was performed using a Perkin Elmer Turbomatrix HS-40
220 trap automatic headspace sampler with trap enrichment. Fermentate aliquots (2.5 mL) were
221 placed in 22 mL PerkinElmer vials with polytetrafluorethylene (PFTE)/silicone septa and the
222 used extraction conditions were those described by Pogačić et al. (2015).

223

224 2.2.4.3. Analysis of volatile compounds using GC-MS

225 Volatiles were analyzed using a Clarus 680 gas chromatograph coupled to a Clarus 600T
226 quadrupole mass spectrometer (PerkinElmer, Courtaboeuf, France) as described in Pogačić et
227 al. (2015) and Leyva Salas et al. (2019). All samples were analyzed in the same GC-MS run.
228 Standards were regularly injected to verify the absence of instrumental drift of the GC-MS
229 system and blank samples (boiled deionized water) were also injected to check for the
230 absence of carry-over.

231 Volatile compounds were identified as described in Pogačić et al. (2015), comparing their
232 retention index and mass spectral data from the NIST 2008 Mass Spectral Library (Scientific
233 Instrument Services, Ringoes, NJ, USA) with those of authentic standards purchased from
234 Sigma Aldrich (St. Quentin Fallavier, France).

235

236 2.2.4.4. Data processing

237 As described by Pogačić et al. (2015), the PerkinElmer Turbomass software, version
238 5.4.2.1617, was used to perform data pre-processing. After conversion of the GC-MS raw
239 data files to netCDF format with Data Bridge (Perkin Elmer, Waltham, Massachusetts, USA),
240 raw data were converted to time- and mass-aligned chromatographic peak areas using the
241 open source XCMS package implemented with the R statistical language (Smith et al., 2006).

242 A heatmap was generated after mean-centered normalization by R packages of pheatmap v.
243 1.0.12 (Kolde, 2019).

244

245 2.2.5. Identification of potential antifungal peptides

246 2.2.5.1. Identification of peptides by nano-LC-MS/MS

247 Mass spectrometry (MS) analysis was adapted from that described by Nyemb-Diop et al.
248 (2016), using a nanoRSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer
249 (Thermo Scientific, San Jose, USA) equipped with a nanoelectrospray ion source. Briefly,
250 samples were first concentrated using a pepMap100 μ -precolumn (C18 column, 300 μ m i.d. \times
251 5 mm length, 5 μ m particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands),
252 before peptide separation was performed on a PepMap RSLC column (C18 column, 75 μ m
253 i.d. \times 150 mm length, 3 μ m particle size, 100 Å pore size; Dionex); column temperature was
254 maintained at 35°C along peptide separation that was performed at a flow rate of 0.3 μ L \cdot min⁻¹
255 using solvent A (2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in
256 deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01%
257 (v/v) TFA in deionized water). The elution gradient was as follows: a first rise from 5 to 35%
258 solvent B over 35 min, followed by a second rise from 35 to 85% solvent B over 2 min.
259 Eluted peptides were directly electrosprayed into the mass spectrometer operating in positive
260 ion mode with a voltage of 1.8 kV. The mass spectra were recorded in full MS mode using the
261 m/z range 250–2000. The resolution of the mass analyzer for a m/z of 200 a.m.u (atomic mass
262 unit) was set to 70,000 in the acquisition method. For each scan, the ten most intense ions
263 were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at
264 m/z of 200 a.m.u and the parent ion was subsequently excluded from MS/MS fragmentation
265 for 15 s. The instrument was externally calibrated according to the supplier's instructions.
266 Peptides were identified from the MS/MS spectra using X!Tandem pipeline software

267 (Langella et al., 2017) The peptide identification database was an in-house database
268 composed of major milk and egg proteins derived from www.uniprot.org (207 proteins in
269 total). Database search parameters were specified as follows: a non-specific enzyme cleavage
270 was selected; a 0.05 Da mass error was allowed for fragment ions while 10 ppm mass error
271 was allowed for parent ions. The phosphorylation of serine and threonine were selected as
272 variable modifications as well as the oxidation of methionine residues. For each identified
273 peptide, a minimum score corresponding to an e-value below 0.05 was considered to be a
274 prerequisite for valid peptide identification. The peptide false discovery rate was calculated by
275 the software to be less than 0.5% using these parameters.

276 2.2.5.2. Bioinformatic analysis to identify potential antifungal peptides

277 Peptides (n=1236) described as possessing an antifungal activity were selected from the
278 following database: ADP (Wang et al., 2004), BIOPEP (Minkiewicz et al., 2008), CAMP
279 (Waghu et al., 2014), EROP (Zamyatnin et al., 2006), MilkAMP (Théolier et al., 2014) and
280 PeptideDB. Then, a Blast between these peptides and those identified by nano-LCMS/MS in
281 the 3 selected fermentates and in the 2 original dairy substrates (non-fermented) was
282 performed to obtain a list of 61 peptides found in fermentates and/or in dairy substrates. After
283 elimination of peptides present in the dairy substrates, a short list of 16 potential antifungal
284 peptides was obtained.

285

286 2.2.5.3 Antifungal assay of peptides isolated from lyophilized cultures and 287 supernatant

288 Sixteen peptides with potential antifungal activity and previously identified by nano-LC-
289 QToF in the selected fermentates were neosynthesized (Biomatik, Ontario, Canada). Their
290 individual antifungal activity against *M. racemosus* UBOCC-A-109155 and *R. mucilaginosus*
291 UBOCC-A-216004 was assessed using an agar well diffusion method. Briefly, the targeted

292 fungi were included at a final concentration of 1×10^4 spores or cells per mL in potato dextrose
293 broth supplemented with 1% agar and the mix poured into 90 mm Petri dishes. Once
294 solidified, six 9-mm-wells were cut per plate and filled with 100 μ L of the peptide solutions
295 prepared as follows: each of the tested peptides was dissolved in sterile distilled water to
296 obtain a 5 mg/mL stock solution and diluted to 2, 1, 0.5 and 0.25 mg/mL. Agar plates were
297 then incubated at 25 °C and the potential antifungal activity evaluated daily for 7 days. When
298 applicable, diameters of inhibition zones were measured.

299 2.3 Statistical analyses

300 Statistical analyses were performed with the Statgraphics Plus software (Statpoint
301 Technologies Inc., Herdon, VA, USA). One-way analysis of variance (ANOVA) was carried
302 out to detect significant differences among means. A Fisher's least significant difference test
303 was applied to compare the mean values.

304

305 3. Results

306 3.1. Identification and quantification of potential antifungal compounds

307 3.1.1. Organic and fatty acids

308 Overall, 31, 32 and 22 compounds among the 56 targeted organic and fatty acids were
309 detected in the *P. jensenii* CIRM-BIA1774, *L. rhamnosus* CIRM-BIA1952 and *M.*
310 *lanceolatus* UBOCC-A-109119 fermentates, respectively. Concerning organic acids, 11 of
311 them were systematically detected in all 3 fermentates (Table 1). For bacterial fermentates, 3
312 organic acids (*i.e.*, propionic, citric and acetic acids), were quantified at concentrations above
313 10 mg/g in the *P. jensenii* fermentate while only 2 (lactic and citric acids) were present above
314 this level in the *L. rhamnosus* fermentate. In addition, 15 molecules were detected at
315 concentrations comprised between the LOQ values and 1 mg/g in the *L. rhamnosus*
316 fermentate versus 10 in the *P. jensenii* fermentate. Other detected molecules could not be

317 quantified as their values were below the LOQ. Concerning the *M. lanceolatus* fermentate, the
318 13 detected organic acids never exceeded 1 mg/mL in concentration except for citric acid.
319 Lactic acid was present at a high concentration (86.018 ± 3.05 mg/g fermentate) in the *L.*
320 *ramnosus* fermentate but absent in both the *P. jensenii* and *M. lanceolatus* fermentates. As
321 expected, propionic acid was only quantified at high levels in the *P. jensenii* fermentate
322 (59.94 ± 21.28 mg/g). Acetic acid concentrations were 16.893 ± 5.676 mg/g in the *P. jensenii*
323 culture, 2.57 ± 0.62 mg/g (approximately 6-fold less) in that of *L. ramnosus* and $0.104 \pm$
324 0.002 mg/g in the *M. lanceolatus* fermentate. For several compounds, including 2-
325 pyrrolidone-5-carboxylic, (S)-2-hydroxy-4-methylpentanoic acid, hydroxyphenyllactic,
326 phenyllactic acid and mevalonolactone, the highest concentrations were observed in the *P.*
327 *jensenii* fermentate. Noteworthy, *L. ramnosus* fermentate contained relatively high
328 concentrations of succinic acid, 5-oxopyrrolidine-2-carboxylic acid, mevalonolactone, (S)-2-
329 hydroxy-4-methylpentanoic acid and benzoic acid.

330 Concerning free fatty acids, 19 were detected in the different fermentates (Table 1). Among
331 them, 12 compounds (butyric, caproic, hydroxyisocaproic, nonanoic, capric, lauric, myristic,
332 pentadecanoic, palmitic, palmitoleic, stearic and oleic acids) were systematically found in the
333 3 fermentates at various concentrations (Table 1). Among these 12 compounds, 1 (heptanoic
334 acid) and 3 (linoleic, nonadecanoic and arachidic acids) compounds were only found in the *L.*
335 *ramnosus* or *P. jensenii* fermentate, respectively, but concentrations were extremely low (< 2
336 $\mu\text{g/g}$). In addition, 7 compounds (caproic, nonanoic, capric, lauric, pentadecanoic, palmitoleic
337 and oleic acids) showed concentrations below $10 \mu\text{g/g}$ or $\mu\text{g/mL}$. Among the 5 remaining
338 compounds identified in all fermentates, quantitative differences were observed (Table 1).
339 Myristic, palmitic and stearic acids were found at intermediate concentrations, *i.e.* ranging
340 from $>10 \mu\text{g/mL}$ myristic acid in the *M. lanceolatus* UBOCC-A-109193 fermentate to ~ 50
341 $\mu\text{g/g}$ palmitic acid in *L. ramnosus* CIRM-BIA1952. Finally, butyric acid was the most

342 abundant free fatty acid in the *M. lanceolatus* UBOCC-A-109193 fermentate (444.7±0.94
343 µg/mL) while hydroxyisocaproic acid was found in *P. jensenii* CIRM-BIA1774 and *L.*
344 *rhamnosus* CIRM-BIA1952 fermentates with concentrations of 537±30 and 222±8 µg/g,
345 respectively (Table 1).

346

347

348 3.1.2. Identification of volatile compounds by head-space trap GC-MS

349 An untargeted approach was used to search for potential antifungal volatile compounds in
350 fermentates using head-space trap coupled to gas chromatography. Thirty-six volatile
351 compounds were detected in the analysed fermentates (Figure 1). Among them, 15, 11 and 10
352 volatile compounds (including propionate and acetate, and butyrate which were accurately
353 quantified using HPLC and GC-FID, respectively) were detected with the highest relative
354 abundance in *P. jensenii* CIRM-BIA1774, *L. rhamnosus* CIRM-BIA1952 and *M. lanceolatus*
355 UBOCC-A-119109 fermentates, respectively (Figure 1). As shown in Figure 1, the *P. jensenii*
356 CIRM-BIA1774 fermentate contained, in addition to propionate and acetate, 20 to 1792 times
357 higher relative abundances of propanoate esters (i.e. butyl propanoate, ethyl propanoate, 2-
358 methyl propanoate and propyl propanoate) and acetate esters (i.e. ethyl acetate, butyl acetate
359 and butanol- 2-methyl-acetate) in comparison to the other fermentates. In contrast, the *L.*
360 *rhamnosus* CIRM-BIA1952 fermentate was characterized by high relative abundances of
361 furans (2-ethyl furan, 2-n-butyl furan, 2-pentyl furan and 2-furamethanol) and several
362 aldehydes (heptanal, octanal, nonanal) and ketones (acetoine, diacetyl, 2-nonanone and 2-
363 undecanone). A different profile was observed for the *M. lanceolatus* fermentate with high
364 relative abundances of butyrate, esters (ethyl butyrate and ethyl hexanoate), aldehydes
365 (benzaldehyde and 4-methyl benzaldehyde), ketones (2-pentanone, 2, 3-pentanedione and
366 diacetyl) and one alcohol (3-methyl 1-butanol).

367

368 3.1.3. Identification of potential antifungal peptides

369 An untargeted approach was applied to search for potential antifungal peptides present in the
370 different fermentates. Regarding the nano-LC-MS/MS analysis, a total of 1040 peptides were
371 identified in the 3 tested fermentates. Among these peptides, 22, 253 and 853 were identified
372 in *P. jensenii* CIRM-BIA1774, *L. rhamnosus* CIRM-BIA1952 and *M. lanceolatus* UBOCC-
373 A-103193 fermentates, respectively. In addition, *P. jensenii* and *L. rhamnosus* fermentates
374 shared 3 and 85 peptides with the *M. lanceolatus* fermentate, respectively. There was no
375 common peptide between *P. jensenii* and *L. rhamnosus* fermentates. After a Blast search
376 against a list of 1236 known antifungal peptides, 16 peptides were neosynthesized for *in vitro*
377 antifungal assays (Table 2). They had E-values ranging from 1.6×10^{-2} to 2×10^{-6} , identities
378 between 47 and 100% and relative abundances between 1.7×10^{-2} and 8.9×10^{-8} . They
379 corresponded to α_{s2} and κ -casein hydrolysates..

380

381 3.1.4. Antifungal activity of identified peptides

382 Out of the 16 peptides evaluated for antifungal activities by the agar diffusion method against
383 *M. racemosus* and *R. mucilaginosa*, only one (pepa4c177 detected in *L. rhamnosus*
384 fermentate) showed significant activity (Figure 2). This peptide contained 9 amino acids and
385 was derived from α_{s2} -casein f(165-203) (Table 2). This peptide inhibited both *M. racemosus*
386 and *R. mucilaginosa* at the highest tested concentrations (2.5 and 5 mg/mL) while only *M.*
387 *racemosus* was inhibited at 1 mg/mL (Figure 2). After 7 days at 25°C, the peptide remained
388 active against the two tested fungi, with inhibition zones of 2 and 3 mm in diameter around
389 wells containing 2 and 5 mg/mL, respectively.

390

391 4. Discussion

392 In this study, 3 fermentates obtained from *L. rhamnosus*, *P. jensenii* and *M. lanceolatus*,
393 previously shown to exhibit promising antifungal activities in dairy products (Garnier et al.,
394 2019), were studied using a combination of analytical methods to identify the antifungal
395 compounds involved in their activity. A large variety of organic and fatty acids, volatile
396 compounds and one antifungal peptide was evidenced providing new insights about the types
397 of molecules involved in the antifungal activities of microorganisms used for biopreservation.
398 As expected, lactic and acetic acids were the most abundant fermentation products for *L.*
399 *rhamnosus* while propionic and acetic acids were linked to *P. jensenii*. Their respective and/or
400 combined antifungal activities are well-known and have been reported many times in the
401 literature (Bian et al., 2016; Gerez et al., 2010; Lind et al., 2007). It is worth mentioning that
402 lactic acid *per se* is not considered as a compound with antifungal activity, given its relatively
403 low pKa (3.9) and the pH encountered in dairy products. Nevertheless, Dagnas et al. (2015)
404 showed that, in combination with acetic acid, both acted in synergy. In contrast to lactic acid,
405 acetic and propionic acids have higher pKa values (pKa =4.75 and 4.87, respectively). Given
406 their high concentrations in the bacterial fermentates, they likely contribute to the observed
407 antifungal activity. Organic acid concentrations in the *M. lanceolatus* fermentate were
408 substantially inferior to the other two, except for citric acid that was quantified at high levels
409 in all fermentates. It is also naturally present in milk. Although the antifungal activity of this
410 acid has been reported, its efficiency is limited when compared to other organics acids such as
411 acetate and benzoate (Hassan et al., 2015; Shokri, 2011). However, it also acts as a precursor
412 for other compounds such as diacetyl which is well known for its antifungal activity as
413 discussed below.

414 The *P. jensenii* fermentate, and to a lesser extent the *L. rhamnosus* one, contained numerous
415 hydroxylated compounds such as 3-(4-hydroxyphenyl) propionic acid, (S)-(-)-2-
416 hydroxyisocaproic acid and DL-hydroxyphenyllactic acid. The hydroxyl group improves acid

417 bioactivity by enhancing viscosity and reactivity as compared to their non-hydroxylated
418 counterpart and by providing an easier partition into the membrane (Pohl et al., 2011). Honoré
419 et al. (2016) showed that these compounds mostly contributed to the antifungal activity of
420 *Lactobacillus paracasei* DGCC 2132. It has also been reported that decanoic acid derived
421 hydroxylated acids inhibited *R. mucilaginosa* growth at concentrations as low as 10 µg/mL
422 (Sjögren et al., 2003). This compound could therefore contribute to the antifungal effect of the
423 *P. jensenii* fermentate. Phenyllactic acid, which was quantified at concentrations of 0.596
424 mg/g in the *P. jensenii* fermentate, has also been shown to be an efficient antifungal
425 compound (Lavermicocca et al., 2003).

426 Free fatty acids were also searched for and quantified. Concerning *L. rhamnosus* and *P.*
427 *jensenii* fermentates, concentrations were low (<0.06 mg/g) for all identified free fatty acids,
428 except for (S)-(-)-2-hydroxyisocaproic acid. Low concentrations were also observed in the *M.*
429 *lanceolatus* fermentate except for butyric acid which had a concentration of 0.537 mg/mL.
430 Butyric acid has been shown to exhibit antifungal activity (Pohl et al., 2011) and may play a
431 significant role in the observed *M. lanceolatus* fermentate antifungal activity. Its presence in
432 high concentration likely results from *M. lanceolatus* lipolytic activity.

433 Because volatile compounds may possess antifungal activity, as recently shown by Aunsbjerg
434 et al. (2015) for diacetyl, they were also analysed in the different fermentates. Diacetyl was
435 present in all 3 fermentates but the highest prevalence was observed in *L. rhamnosus* and *M.*
436 *racemosus* fermentates. This compound was shown to play a non-negligible role in the
437 antifungal activity of *L. paracasei* DGCC 2132 against two *Penicillium* spp. strains
438 (Aunsbjerg et al., 2015). Similarly, ethyl acetate was found in all fermentates and was
439 previously shown to be the main compound responsible of the antifungal activity of
440 *Wickerhanomyces anomalus* in sourdough bread (Coda et al., 2011). More recently, 2-methyl
441 propanoate, present at a high relative abundance in the *P. jensenii* fermentate, showed

442 increased antifungal activity against *Fusarium culmorum* and *Cochliobolus sativus* (Kaddes et
443 al., 2019). However, to our best knowledge, the antifungal activity of most of the identified
444 volatile compounds is not known. Consequently, it could be of interest to test their potential
445 antifungal activity, alone or in combinations and, if active, to accurately quantify them.

446 Peptides can also be involved in the antifungal activity of biocontrol agents (Leyva Salas et
447 al., 2017), therefore, an untargeted approach was used to identify such compounds. One basic
448 peptide, pepa4c177 (RLNFLKKIS), from α_{s2} -casein f(165-203) and identified in the *L.*
449 *rhamnosus* CIRM-BIA1952 fermentate, showed clear *in vitro* antifungal activity against *R.*
450 *mucilaginosa* and *M. racemosus*, during the seven day evaluation at 2 and 1 mg/mL,
451 respectively. The C-terminal region of this peptide is undoubtedly essential for its antifungal
452 effect since the pepa4b61, pepa4c101 and pepa4b79 peptides, whose sequences partially
453 overlapped with pepa4c177, turned out to be inactive. It would be necessary to quantify
454 pepa4c177 in the fermentate to define whether it takes part, or not, in the observed *in situ*
455 antifungal activity. Noteworthy, this antifungal peptide is not *de novo* synthesized by
456 *L. rhamnosus* but results from its proteolytic activities. In most studies on antifungal LAB,
457 cyclic dipeptides are the main peptides related to the observed antifungal activity. Their
458 production by *Lactobacillus* species has already been reported (Magnusson et al., 2003; Ström
459 et al., 2002). A MIC of 20 mg/mL was determined for *L. plantarum* cyclo(L-Phe-L-Pro)
460 against *Aspergillus fumigatus* and *Penicillium roqueforti* (Ström et al., 2002), which is 10
461 times higher than that observed for pepa4c177. On the other hand, cyclo(L-Pro-D-Leu)
462 produced by a *Bacillus cereus* subsp. *thuringiensis* strain had a MIC of 8 μ g/mL against
463 *Aspergillus flavus* (Nishanth Kumar et al., 2013). It would also be interesting to further
464 investigate the action spectrum and MIC of the pepa4c177 peptide as it is a novel peptide
465 described for the first time in the present study. Noteworthy, *L. rhamnosus* CIRM-BIA1952
466 antifungal activity was previously evaluated as an adjunct bioprotective culture in different

467 dairy product models (Leyva-Salas et al. 2018). Despite it showed, to some extent, a slight
468 antifungal activity in a yogurt model, its activity was far less pronounced than for other tested
469 *L. rhamnosus* strains (Leyva-Salas et al. 2018).

470 Overall, more than fifty compounds were identified in the 3 fermentates. It is reasonable to
471 assume that they were not equally involved in the antifungal activity observed against *R.*
472 *mucilaginoso* and *M. racemosus*. Moreover, synergistic and/or additive effects between
473 compounds have been reported and make it more complex to understand their mechanism of
474 action (Crowley et al., 2013; Suomalainen and Mäyrä-Makinen, 1999). To clarify this aspect,
475 MIC determinations for each compound identified as well as their MIC in a mixture against
476 the two fungi could be conducted.

477 The results reported in this study provide new insights to better understand the antifungal
478 activities of bacterial and fungal fermentates. Further work will be necessary to precisely
479 identify which molecules are the main actors involved in the observed antifungal activities of
480 the studied fermentates, and how these molecules act and affect fungal cell physiology at a
481 cellular level.

482

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620

621 **Table 1. Organic and fatty acids with known antifungal activity detected and/or quantified in antifungal fermentates.**

Compound (common name)	Analysis method*	Fermentate		
		<i>P. jensenii</i> CIRM-BIA1774 (µg/g)	<i>L. rhamnosus</i> CIRM-BIA1952 (µg/g)	<i>M. lanceolatus</i> UBOCC-A-109193 (µg/mL)
Organic acids				
Ethanoic acid (acetic acid)	1	1.69x10⁴ ± 0.57x10⁴^a	2.57x10 ³ ± 0.62x10 ³ ^b	1.04x10 ² ± 2 ^c
Propanoic acid (propionic acid)	1	5.99x10⁴ ± 2.128x10⁴^a	9.99x10 ² ± 8.9x10 ² ^b	-
2-Hydroxypropanoic acid (DL-lactic acid)	1	-	8.60x10⁴ ± 0.3x10⁴	-
Butanedioic acid (succinic acid)	1	4.85x10 ³ ± 2.78x10 ³ ^a	5.22x10³ ± 0.5x10³^a	99 ± 24 ^b
5-Oxopyrrolidine-2-carboxylic acid (2-pyrrolidone-5-carboxylic acid)	2	2.23x10³ ± 0.33x10³^a	1.8x10 ² ± 87 ^b	1.1 ± 0 ^c
(4S)-4-hydroxy-4-methylxan-2-one (mevalonolactone)	2	4.82x10² ± 16^a	1.51x10 ² ± 7 ^b	+
2-Hydroxypropane-1,2,3-tricarboxylic acid (citric acid)	1	3.25x10⁴ ± 0.5x10⁴^a	1.45x10 ⁴ ± 0.13x10 ⁴ ^b	2.51x10 ³ ± 30 ^c
Benzoic acid	1	4 ± 1 ^c	1.54x10² ± 5^a	17.1 ± 0.2 ^b
2-Hydroxybenzoic acid (salicylic acid)	2	0.5 ± 0 ^a	0.6 ± 0.1 ^a	+
3-Phenylpropanoic acid (hydrocinnamic acid)	2	+	+	+
3-(4-hydroxyphenyl)propanoic acid	2	4.95x10² ± 22^a	13 ± 0.4 ^b	-
(S)-2-Hydroxy-3-phenylpropanoic acid (phenyllactic acid)	2	5.96x10² ± 24^a	12 ± 1 ^b	12 ± 0.1 ^b
2-Hydroxy-3-(4-hydroxyphenyl)propanoic acid (hydroxyphenyllactic acid)	2	3.12x10² ± 12.5^a	11 ± 1 ^b	-
Nonanedioic acid (azelaic acid)	2	-	+	0.5 ± 0.1
Fatty acids				
Butyric acid	3	8.7 ± 2.44 ^b	6.6 ± 0.70 ^b	4.45x10² ± 0.94^a
Caproic acid	3	1.2 ± 0.51 ^b	1.4 ± 0.40 ^b	6.3 ± 0.22^a
Heptanoic acid	3	-	0.1 ± 0.20	-

(S)-2-Hydroxy-4-methylpentanoic acid ((S)-(-)-2-hydroxyisocaproic acid)	2	5.37x10² ± 30^a	2.22x10 ² ± 8 ^b	0.7 ± 0.1 ^c
Caprylic acid	3	0.8±0.75 ^a	1.4±0.10^a	-
Nonanoic acid	3	1.1±0.09 ^b	1.8±0.30^a	1.4±0.09 ^a
Capric acid	3	1.6±0.50 ^c	3.0±0.30 ^b	4.5±0.25^a
3-Hydroxydecanoic acid	2	+	+	+
Lauric acid	3	2.7±0.17 ^c	5.0±0.20^a	3.1±0.01 ^b
2-Hydroxydodecanoic acid (2-hydroxylauric acid)	2	-	+	-
3-Hydroxydodecanoic acid (3-hydroxylauric acid)	2	+	+	+
Tridecanoic acid	3	1.5±0.20^a	0.3±0.30 ^b	-
Myristic acid	3	2.8±1.05 ^b	9.7±0.80 ^a	11.0±6.69^{ab}
Pentadecanoic acid	3	1.8±0.67 ^a	1.1±0.10 ^a	4.1±2.78^a
Palmitic acid	3	21.0±5.65 ^b	49.9±5.60^a	31.9±15.94 ^{ab}
Palmitoleic acid	3	0.9±0.89 ^a	1.6±1.00 ^a	6.6±4.85^a
Stearic acid	3	13.2±5.02 ^b	32.0±3.40^a	8.5±3.13 ^b
Oleic acid	3	0.6±1.00 ^b	8.8±1.30^a	5.8±2.52 ^a
Linoleic acid	3	0.3±0.46	-	-
Nonadecanoic acid	3	0.9±1.48	-	-
Arachidic acid	3	1.7±2.88	-	-

622 Results are expressed as the mean of three replicates ± standard deviation. Within a same line, means with different letters are significantly different according to a Fisher's
623 least significant difference test ($p < 0.05$).

624 Legend: (+) value between LOD and LOQ; (-) molecule not detected or at concentration < LOD. Bold values correspond to the highest concentration among the three
625 analyzed fermentates (bold characters correspond to the highest observed values). Identification and quantification method: (1) HPLC coupled to UV or refractometer
626 detectors; (2) LC-Q-ToF; (3) GC-FID.

Table 2. Peptides identified in the studied fermentates and tested for their antifungal activity

Peptide	Amino acid sequence	Length	Charge	Protein	Fermentate
pepa4c177	RLNFLKKIS	9	3	α_{s2} -casein f(165-203)	<i>L. rhamnosus</i>
pepa4c223	YLKTVYQHQ	9	2	α_{s2} -casein f(165-203)	<i>L. rhamnosus</i>
pepa5b99	QKPVALINNQFLPYPPYAKPA	21	1	κ -casein f(43-97)	<i>L. rhamnosus</i>
pepa5b83	PYYAKPAAVRSPAQILQWQVL	21	2	κ -casein f(43-97)	<i>L. rhamnosus</i>
pepa5c104	QQKPVALINNQFLPYPPYAKPA	22	1	κ -casein f(43-97)	<i>L. rhamnosus</i>
pepa5c165	YYQQKPVALINNQFLPYPPYAKPAA	25	2	κ -casein f(43-97)	<i>L. rhamnosus</i>
pepa4b78	KLTEEEKNR	9	0	α_{s2} -casein f(165-203)	<i>M. lanceolatus</i>
pepac192	TKLTEEEKNR	10	0	α_{s2} -casein f(165-203)	<i>M. lanceolatus</i>
pepa4b97	KTKLTEEEKNR	11	1	α_{s2} -casein f(165-203)	<i>M. lanceolatus</i>
pepa4b61	LTEEEKNRLNF	11	-1	α_{s2} -casein f(165-203)	<i>M. lanceolatus</i>
pepa4b98	KTKLTEEEKNRL	12	1	α_{s2} -casein f(165-203)	<i>M. lanceolatus</i>
pepa4c197	TKKTKLTEEEKNR	13	2	α_{s2} -casein f(165-203)	<i>M. lanceolatus</i>
pepa4c190	STEVFTKKTCLTEEEKNR	18	1	α_{s2} -casein f(165-203)	<i>M. lanceolatus</i>
pepa4b79	KLTEEEKNRLNFL	13	0	α_{s2} -casein f(165-203)	<i>P. jensenii</i>
pepa4c101	KTKLTEEEKNRLNFLK	16	2	α_{s2} -casein f(165-203)	<i>P. jensenii</i>
pepa5b48	LINNQFLPYPPYAKPA	16	1	κ -casein f(43-97)	<i>P. jensenii</i>

Figure legends.

Figure 1. Mean-centered normalized heatmap showing the main volatile compounds present in the studied antifungal fermentates (relative abundance of each compound is shown in brackets).

Figure 2. Antifungal activity of peptide pepa4c177 on *Rhodotorula mucilaginosa* (A) and *Mucor racemosus* (B) grown in potato dextrose broth supplemented with 1% agar at 25°C for 7 days. Five concentrations were tested 0.25, 0.5, 1.0, 2.0 and 5 mg/mL. Central wells were filled with sterile water as negative control.

Figure 1.

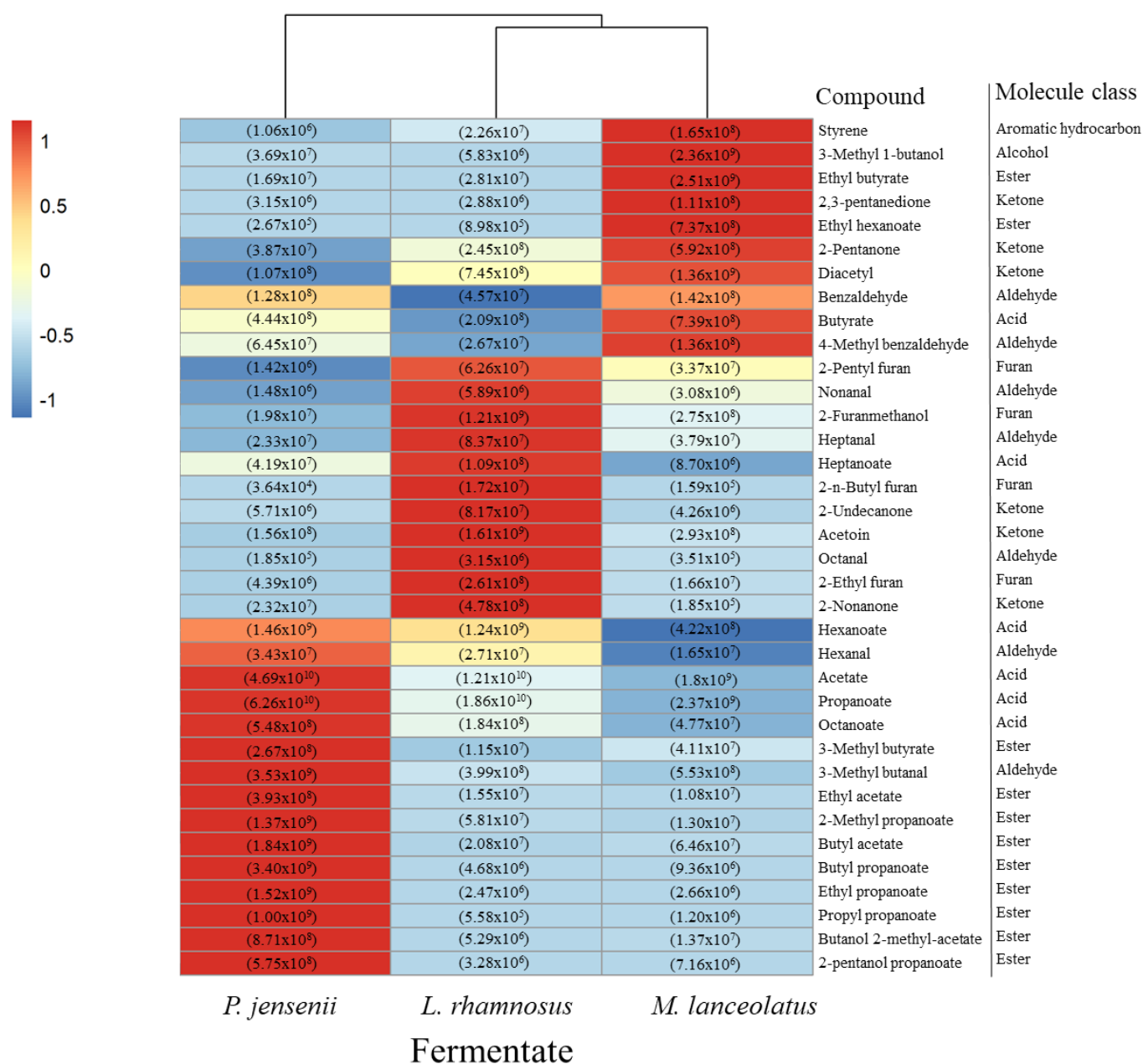


Figure 2.

